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Specification and Drawing, as originally filed, with Application for Patent Serial No:
2,393,726, on **July 16, 2002**, by **STEVEN J. LOCKE AND DEVANAND PINTO**, for
"Quantitative Proteomics Via Isotopically Differentiated Derivatization". The said
invention was made while your Petitioners were employed as a public servants as defined
in the Public Servants Inventions Act in **National Research Council of Canada**, and
pursuant to Section 5 of that Act, the said invention has been determined to be vested in
National Research Council of Canada.

L. Legimbal
Agent certificateur / Certifying Officer

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Abstract

A method for the quantitative analysis of a plurality of samples containing amines, particularly primary or secondary amines, by mass spectrometry is disclosed. The method involves the chemical derivatization of amines in the sample by reacting the samples with isotopically labelled reagents, resulting in the isotopic labelling of the alkylamine derivatives. The method allows for the simultaneous examination and analysis of a plurality of samples. The method is particularly useful in the field of proteomics.

QUANTITATIVE PROTEOMICS VIA ISOTOPICALLY DIFFERENTIATED DERIVATIZATION

Field of the Invention

[0001] The field of the invention is quantitative proteomics coupled with mass spectrometry. The invention also relates to the quantitative analysis of amine-containing molecules by mass spectrometry.

Background of the Invention

[0002] The emerging field of proteomics involves the characterization of the total protein repertoire of a sample. As there are numerous proteins in most samples, proteomics involves a high-throughput analysis and relies on the integration of significant advances recently achieved in two-dimensional (2D) gel electrophoretic separation of proteins, mass spectrometry and bioinformatics. As a result of these advances, it is now possible to obtain profiles of protein expression with much greater sensitivity and reproducibility. However, these analyses, though fast and easily automated, are not reliably quantitative.

[0003] Most present quantitative methods suffer from a major flaw, which may also affect qualitative comparisons, arising from lack of adequate reproducibility of 1-D and 2-D gel electrophoresis, or of other separation techniques for proteins such as immuno-affinity chromatography. It is very difficult to make two 2-D gel plates, or two chromatography columns, behave in exactly the same way. Therefore, when one compares cells from two samples (for example in a normal healthy state with those in a diseased state), one has to run the two protein extracts on two different gels (or columns, or at different times on the same column) whose characteristics can be significantly different despite advances in these technologies.

[0004] Some recent advances in this science allow the protein extracts from two samples to be run on the same gel or column at the same time, thus avoiding all the ambiguities arising from variability in the separation procedure. However, these methods suffer from limitations in other areas.

[0005] For example, Oda et al., (1999) describe a method whereby two cell cultures can be compared. The cells are cultured on growth media isotopically enriched by ¹⁵N-leucine.

This method has the advantage that no chemical derivatization is required, and the two cultures to be compared can be mixed (and thus subjected to the identical conditions) before any of the subsequent analytical processing is done. However, it is applicable only to cases in which the appropriate cells can be grown in culture and is not applicable to larger organisms. Furthermore, it is limited to comparing two cultures.

[0006] Geng et al., (2000) describe a method using a lectin column to select glycoproteins from different proteomes which are subsequently labelled by acetylation of amino groups with N-acetoxysuccinimide or d_3 -C¹-N-acetoxysuccinimide. However this method involves separate digestion of the two samples prior to labelling which can affect quantitation. In addition, the basic character of the peptide lysines are masked by the labelling reaction. This results in the positive ion spectra being dominated by Na⁺ and K⁺ adducts and sequencing by MS/MS is more difficult. Furthermore, the method disclosed is limited to comparing two samples.

[0007] Ji et al., (2000) describe a method using immobilized metal affinity chromatography after isotopic labelling to select for histidine-containing peptides. However, this method is limited to histidine residues, which are not very abundant (the abundance of histidine is estimated to be approximately 2%). In addition, this method can result in loss of post-translational modifications. Numerous proteins are regulated by post-translational modifications.

[0008] Mirgorodskaya et al., (2000) discloses a method involving a comparison of the MALDI intensity of a protein sample hydrolysed by chymotrypsin-like duodenase in ¹⁶O-water to a known concentration of the same sample that has been hydrolysed in ¹⁸O water to produce ¹⁸O-labelled internal standards. However, this technique is limited in that it requires separate digestion of the two samples which can affect quantitation. Another limitation of methods that use ¹⁸O water is the potential for exchange of other oxygen groups in the peptide after the initial reaction with trypsin. This results in additional peaks appearing for one peptide. As a result, the sensitivity decreases and the complexity of the spectrum increases which can affect the accuracy of the quantitation. Furthermore, the method disclosed is limited to comparing two samples. Yao et al., (2001) disclose a similar method employing trypsin.

[0009] Munchbach et al., (2001) describe a method of selective N-terminal acylation of peptides with 1-([d₀-or d₄]-nicotinoloxo) succinimide esters. The process involves initial blockage of the ε-amino of protein lysines with succinic anhydride followed by enzymatic digestion with Asp (Glu) C protease. The peptides produced are selectively N-terminal acetylated with one of the isotopically labelled succinimide esters and then analysed by electrospray or MALDI. However, this technique masks the basic nature of the lysine residues. The basicity of the lysine residue is a critical parameter for isoelectric focusing (the first step in 2D gel electrophoresis), therefore, any labelling process should aim to minimize the change in basicity between the native and labelled lysine. This method converts the lysine from a primary amine to an amide with an attached pyridine, thus greatly altering the basicity of lysine. This will have a large effect on the isoelectric focusing of the protein. As a result of this drawback, labelling was done after 2D gel separation (each sample had to be run on a separate gel), which can affect the accuracy of the quantitation. Furthermore, the method disclosed is limited to comparing two samples.

[0010] Goodlett et al., (2001) disclose a method whereby the protein mixtures from two different samples are enzymatically hydrolysed with trypsin, followed by the labelling of the resultant peptides with either d₀ or d₃-methanol. However, this method labels the peptides after digestion, and although the basic character of the lysine remains, the acidic nature of the aspartic/glutamic acid is lost. Furthermore, the method disclosed is limited to comparing two samples.

[0011] Smith et al., (2001) employs high performance Fourier transform-ion cyclotron resonance (FTICR) mass spectrometry to generate accurate mass tags for peptides or intact proteins. The principle is that the accuracy of the mass measurement will be such that the mass of the peptide is unique amongst all the other peptide masses predicted for a genome. However, this method relies on the high mass accuracy attainable with the FTICR mass spectrometer which is very costly.

[0012] Three recent papers describe methods of quantitative proteomics via cysteine-residue labelling.

[0013] Gygi et al., (1999) describe a method of chemical derivatization using cysteine residues as the targets. This approach involves differential labelling of the native proteins immediately after extraction of the protein complement from the samples, followed by mixing of the samples to be compared before any further steps are performed. However, the particular derivatization reaction employed involves chemical attachment of biotin groups to cysteine residues in the proteins, which can result in loss of potentially important post-translational modifications, particularly phosphorylation. Further, cysteine residues are relatively rare in protein samples. This method was developed specifically for use with affinity purification rather than 1-D or 2-D gel electrophoresis, and one can foresee problems in its application to these important separation techniques. Furthermore, this method allows only two samples to be compared.

[0014] Wang et al., (2001) discloses a method involving the N-acylation of peptides with d_0 or d_4 -succininic anhydride following the separate tryptic digestion of protein samples. Cysteine-containing peptides are selected by covalent chromatography based on thiol-disulfide exchange with a thiopropyl Sepharose gel. However, this method suffers from the same limitations as Gygi et al. (1999) and only two samples can be compared.

[0015] Conrads et al., (2001) uses $^{14}\text{N}/^{15}\text{N}$ metabolic labelling but combines this with biotin labelling of cysteine residues, in a similar manner to Gygi et al., (1999). Again, the method suffers from the same limitations as Gygi et al., (1999) and only two samples can be compared.

[0016] Three recent papers describe methods of protein quantification using phosphoprotein-selective reagents and methods.

[0017] For example, Weckwerth et al., (2000) presents a method for the relative quantitation of phosphoproteins to compare the phosphorylation status of proteins under two different states. The method involves chemical treatment of the proteins to selectively remove phosphate from phospho-Ser/Thr followed by addition of ethanethiol or ethane- d_5 -thiol. However, the isotopically-specific chemical derivatization occurs at a late stage in the analysis (following proteolysis of the proteins). In addition, there are too many steps in which the two protein extracts are processed separately, and then made to react with

chemical and biochemical reagents in different reaction mixtures whose reproducibility cannot be guaranteed. Thus, the relative abundances of target proteins derived from such procedures are liable to systematic errors of unknown magnitude in any given case. Furthermore, the method disclosed is limited to comparing two samples.

[0018] Molloy et al., (2001) describes a similar method to Goodlett et al., (2001), except that a mixture of two different non-isotopically labelled alkanethiols is used for the qualitative detection of phosphopeptides. However, this method is selective for phosphoproteins. Furthermore, the method disclosed is limited to comparing two samples.

[0019] Finally, Goshe et al., (2001) presents a method combining stable isotope and biotin labelling to enrich and quantitatively measure differences in the O-phosphorylation states of proteins. However, this method is selective for phosphoproteins and only two samples can be compared.

[0020] Thus, although there have been some recent attempts at quantitative proteomics, these approaches are limited in that (1) if the proteins are tagged or labelled, the methods are limited to comparing only two samples, (2) the approaches are too cumbersome and problematic, (3) the approaches involve labelling cysteine residues, which are generally rare in protein samples, and which can result in loss of post translational modifications, (4) they are limited to affinity purification, (5) they are limited to samples that can be cultured *in vitro*, (6) they require use of a costly mass spectrometer and (7) they are limited to detection of phosphoproteins.

[0021] Thus, there is a need to develop an effective method of quantitative proteomics which allows for (1) simple and easy labelling of the sample, (2) comparison of more than two samples simultaneously, (3) simultaneous preparation of two or more samples to avoid variability between samples, (4) labelling residues that are common and not involved in post-translational modification, (5) use of any method of protein separation, including 1-D and 2-D gel electrophoresis, (6) analysis of any sample, and is not limited to samples that can be cultured *in vitro*, and (7) use of an inexpensive mass spectrometer (MS).

Summary of the Invention

[0022] According to one aspect of the present invention, there is provided a method for the simultaneous quantitative analysis of a plurality of samples of amine-containing molecules, comprising: (i) reacting the samples with isotopically labelled reagents, wherein the reagents react with the amines, resulting in isotopically labelled derivatives, (ii) combining the samples, and (iii) examining the samples by mass spectrometry.

[0023] According to another aspect of the present invention there is provided a method for the quantitative analysis of a sample of amine-containing molecules, comprising (i) reacting the sample with isotopically labelled reagents resulting in the reductive alkylation of the amines to their alkylamine derivatives, such that the alkylamine derivatives are isotopically labelled and (ii) examining the sample by mass spectrometry.

[0024] According to another aspect of the present invention there is provided a method of for the quantitative analysis of a sample of amine-containing molecules, comprising (i) reacting the sample with isotopically labelled reagents resulting in the reductive alkylation of the amines to their alkylamine derivatives, such that the alkylamine derivatives are isotopically labelled, (ii) combining the samples, and (iii) examining the fragments by mass spectrometry. Optionally, the method comprises the additional step of cleaving the samples into fragments, prior to examining the samples by mass spectrometry. The fragments can be introduced into a mass spectrometer using electrospray ionisation, for example nanospray, pneumatically assisted electrospray, ionspray and turboionspray. Optionally, the method can comprise one or more additional steps of denaturing the samples prior to reacting the samples with different isotopically labelled reagents, separating the amine-containing molecules from the samples after combining the samples, separating the fragments after enzyme-cleaving the samples and sequencing the fragments.

[0025] The step of separating the amine-containing molecules can be selected from a group consisting of 1-D gel electrophoresis (such as SDS-PAGE or isoelectric focusing), 2-D gel electrophoresis, zone electrophoresis, isotachopheresis, ion exchange chromatography, normal phase chromatography, reverse phase chromatography, hydrophobic interaction chromatography, size exclusion chromatography and combinations thereof. The step of separating the fragments after the enzyme digestion can be selected from a group consisting

of liquid chromatography, high performance liquid chromatography and capillary electrophoresis. Analysis of the fragments can be done by techniques such as collision-induced dissociation, post-source decay, multi-photon dissociation, peptide mass fingerprinting, peptide mapping, Edman sequencing and sequencing by sequential amino acid cleavage. Using computer-searching algorithms, the data can be correlated with sequences listed in sequence databases to identify the molecule from which the sequenced fragment originated.

[0026] Optionally, the isotopically labelled reagents are an aldehyde and a reducing agent wherein the aldehyde is selected from a group consisting of formaldehyde and acetaldehyde and the reducing agent is selected from a group consisting of a sodium cyanoborohydride, sodium borohydride, dialkyl borane complexes and pyridine borane complexes.

[0027] The samples can be selected from a group consisting of cells, cellular extracts, sub-cellular extracts, cellular lysates, peptides, proteins, drugs, toxins, antibodies and pollutants. Most often the sample comprises proteins.

[0028] The mass spectrometer can be selected from a group consisting of Fourier transform – Ion cyclotron resonance mass spectrometers (FT-ICR-MS), Time of Flight mass spectrometers (TOF-MS, TOF-TOF-MS), Ion trap mass spectrometers (IT), Quadrupole mass spectrometers (Q-MS and QqQ-MS), Ion mobility mass spectrometers (IM-MS), Quadrupole (or hexapole, octapole)-Time of Flight mass spectrometers (Q-TOF, and Qq-TOF) and Ion trap – Time of flight mass spectrometers (IT-TOF). The mass spectrometer can be combined with an ionization source, for example electrospray ionization, matrix-assisted laser desorption and ionization (MALDI), field desorption, thermal desorption and laser desorption.

[0029] According to another aspect of the present invention, there is provided the use of a mass spectrometer for the analysis of a samples, using the method for the quantitative analysis of a sample of amine-containing molecules, comprising: (i) reacting the entire native sample with isotopically labelled reagents resulting in isotopically labelled derivatives and (ii) analysing the sample by mass spectrometry.

[0030] According to another aspect of the present invention there is provided a kit comprising isotopically labelled reagents, software and protocols for the method of quantitative analysis of a sample of amine-containing molecules, comprising: (i) reacting the entire native sample with isotopically labelled reagents resulting in isotopically labelled derivatives and (ii) analysing the sample by mass spectrometry.

[0031] According to another aspect of the present invention, there is provide a method for the quantitative analysis of two or more cellular extracts of amine-containing molecule, comprising (i) reacting the extracts with different isotopically labelled reagents resulting in the reductive alkylation of the amines to their alkylamine derivatives, such that the alkylamine derivatives are isotopically labelled, (ii) combining the extracts, (iii) separating the amine-containing molecules, (iv) enzymatically cleaving the amine-containing molecules into fragments, (v) separating the fragments, (vi) analysing the fragments by mass spectrometry, and (vii) sequencing the fragments.

Brief description of the Figures

[0032] Figure 1 is a MS spectrum of the same protein from two samples, where each sample was labelled with a unique tag, and digested with chymotrypsin and analysed using ESI-MS.

[0033] Figure 2 is a MS spectrum as in Figure 1, except that three samples were labelled. In addition the peptides were triply charged.

[0034] Figure 3 is a LC- MS spectrum demonstrating that the tags are compatible with gel electrophoresis and also demonstrating the added specificity obtained through labelling

[0035] Figure 4 is a tandem MS spectrum of two peptides with isotopic labelling.

[0036] Figure 5 is a composite of 4 spectrums. Top left, nanoelectrospray spectrum; bottom left, total-ion chromatogram for the m/z range of 475-485; top right, MS spectrum for the first peak consisting of a triplet of doubly charged peptides; bottom right, MS spectrum for a second peak consisting of a triplet of triply charged peptides.

[0037] Figure 6 is a MS spectrum of two samples of BSA differentially labelled, combined, digested using trypsin and analysed by LC-MS. Trace A shows the MS spectrum of a pair of labelled tryptic peptides and Trace B shows the extracted ion chromatograms for the same two peptides.

[0038] Figure 7 is a 2D gel separation of membrane proteins from *Aeromonas salmonicida*. Panel A is a separation of proteins from one sample. Panel B is a separation of proteins from two samples grown under different conditions.

[0039] Figure 8 is a composite of the molecular structures of various amines differentially labelled and analysed by MS and a mass spectrum of one of the analysed amines, 3-aminothiophenol.

[0040] Figure 9 is a MS spectrum of a plurality of amine containing molecules differentially labelled and analyzed by MALDI-MS.

Definitions

[0041] Amine-containing molecule means any molecule containing at least one amine. Preferably the molecule contains a primary or secondary amine.

[0042] CID means collision induced dissociation.

[0043] Electrospray means a source of ionization in which a liquid sample is nebulized from a tube due to the sufficiently high potential applied, with or without a nebulizing gas, which also provides a charge to the droplet, and in which the resultant charged droplet evaporates and fragments yielding small charged droplets or charged molecular ions.

[0044] ESI-MS means electrospray ionization mass spectrometer.

[0045] FTICR means Fourier transform – Ion cyclotron resonance mass spectrometer.

[0046] HPLC-MS means high performance liquid chromatography coupled with a mass spectrometer.

[0047] IM-MS means ion mobility mass spectrometer.

[0048] IT-MS means ion trap mass spectrometer.

[0049] Laser Desorption Ionization means a source of ionization in which ions are produced from a sample adsorbed onto a surface, such as silicon, by exposure to laser irradiation, typically at low pressure (although recently atmospheric pressure is being used).

[0050] MALDI means a source of ionization (Matrix Assisted Laser Desorption Ionization) in which ions are produced from a sample mixed with a matrix (typically analysed in crystalline form) by exposure to laser irradiation, typically at low pressure (although recently atmospheric pressure is being used).

[0051] MS/MS means tandem mass spectrometry, in which ions are selected, caused to fragment and the fragments analysed. It can therefore be used for structural and sequencing studies.

[0052] m/z means the mass to charge ratio.

[0053] Plurality means three or more.

[0054] QMS or QqMS means quadrupole mass spectrometer.

[0055] QToF-MS means quadrupole time of flight mass spectrometer.

[0056] Sample means any composition containing or suspected of containing a substance being identified and measured in an analysis. The sample may contain an amine-containing molecule.

[0057] Sequencing means obtaining the sequence of a protein or protein fragment through correlation of a measured value with values derived from a database or directly through fragmentation in a mass spectrometer.

[0058] ToF-MS means Time of flight mass spectrometer.

Detailed description of the invention

[0059] The invention is an approach to quantitative analysis of any sample based on chemical derivatization with a well-characterized and selective reactivity to achieve the

introduction of an isotopically labelled tag. The invention is particularly useful for proteome analysis. The method involves the reductive alkylation of amine groups to their alkylamine derivatives by the action of an added aldehyde (for example formaldehyde) and a reducing agent, optionally a Schiff base reducing agent, for example sodium cyanoborohydride. This occurs mostly on the side chains of the amino acid lysine, but can also occur on the N-terminal amino group of the protein. The labelled tags are electrophoretically and chromatographically similar but have a sufficient mass shift to be able to easily differentiate them by a mass spectrometer.

[0060] There are many benefits to this invention. Among them are the following:

[0061] The method is applicable to virtually any sample of amine-containing molecules. The most common use envisioned is for protein samples, and in particular use in the field of proteomics. The amino acid lysine is present in almost all proteins. Typically, lysine comprises 3-8% of the total amino acid content of proteins. For example, bovine serum albumin (BSA, about 67 kDa), contains 60 lysine residues per molecule. The reductive alkylation and enzymatic cleavage generates a specific pattern of labelled peptides for quantitation. The specific patterns produced greatly simplifies the analysis of the samples by mass spectrometry. The presence of multiple representative peptides from each protein provides a high degree of quantitative accuracy and increases the ability of measuring low-abundance proteins.

[0062] More than two samples can be analysed simultaneously. The method provides a minimum of eight distinct labels, thus enabling eight different samples, such as eight extracts, to be probed simultaneously. This can be accomplished by differentially labelling two reagents, using both hydrogen and carbon isotopes (for example, as shown in Table 1), which results in eight different masses of the chemically derivatized products. Existing methods allow only two states to be probed at a time. This is because most reagents used for protein quantitation consist of a light tag (natural abundance isotopes) and heavy tag (fully deuterated) since synthesis of tags that are partially deuterated is difficult. In addition, due to the low throughput of current proteomic technologies, many labs are finding the comparison of more than two samples unmanageable. The technology we describe uses two isotopically labelled reagents. This combination enables the number of samples to be

doubled, i.e. each aldehyde can be used with one of two different reducing agents. Therefore, it is possible to expand the number of labels even further by using radioactive formaldehyde and/or cyanoborohydride. Most other labelling methods involve the use of only one isotopically labelled reagent.

[0063] The present invention uses isotope-labelled chemical derivatization of the entire protein extracts, in a way which is generally applicable, and not intrinsically limited to cultured cells. The entire extracts to be compared are derivatized with reagents which are chemically identical but have different stable isotope contents (typically deuterium). This is done prior to mixing of these different derivatized extracts, separation of the constituent proteins (now mixtures because of the different labelling), proteolytic digestion and analysis by mass spectrometry.

[0064] The technique allows for the simultaneous separation and detection of the samples containing the isotopic tags. The basis of the new advance is to run the protein extracts from two (or more) samples on the same gel or column at the same time, thus avoiding all inaccuracies arising from variability in the separation procedures. The method mixes together the protein extracts to be compared after the protein extraction and chemical derivatization steps. Both of these steps are essentially 100% efficient so that no distortion of relative abundance can arise.

[0065] The invention involves derivatization of lysine residues, which are generally more numerous than the cysteine residues targeted in the method described in Gygi et al., (1999). Lysine residues are generally also more accessible to chemical reagents as they are less likely to be buried in the hydrophobic core of a protein in aqueous solution. In addition, lysine residues are more likely to be in a reactive form unlike cysteines which typically exist in a disulphide form that must be reduced prior to derivatization.

[0066] As a result of the multiple-labelling capability, the invention can distinguish among more samples in a single experiment than can the method in Gygi et al., (1999), conferring a proteomic advantage. Also, due to the choice of lysine rather than cysteine residues as derivatization targets, generally the present method yields more labelled proteolytic

peptides per protein molecule, with a consequent increase in sensitivity and accuracy of quantitation (an analytical advantage).

[0067] The chemistry of the derivatization is established and essentially quantitative under the conditions established in the invention. For formaldehyde and sodium cyanoborohydride, each lysine is labelled with two methyl groups under mild reaction conditions. No other amino acids can react with these reagents under the conditions employed, other than ornithine which is one of the very uncommon amino acids with a side chain group similar to that of lysine.

[0068] The molecular mass and isoelectric point of the labelled proteins are not significantly different from those of the unlabelled protein. For example, for BSA (bovine serum albumin), labelling of all lysines with formaldehyde results in a 2.5% increase in the molecular weight. This difference in both molecular weight and pI becomes even less significant for proteins labelled with different isotopes of the same reagent. Thus, the differentially labelled forms of a particular protein from a number of samples co-electrophorese, meaning the different forms of a protein are indistinguishable by 1D or 2D gel electrophoresis. In addition, the differentially labelled samples have high chromatographic similarity. Due to the nature of the labels developed, the chromatographic behaviour of the differentially labelled peptides is very similar. Therefore, the labelled peptides co-elute during LC separation. This is important for accurate quantitation of the samples, since peptide intensity in LC-MS analysis is dependant on sample conditions at the time of elution. In other words, relative quantitation requires that the peptides co-elute, which is not the case for other reagents.

[0069] The presence of a multiplet of peaks of predetermined spacing in the mass spectrum is easily detected and characteristic of peptides from the protein in different samples. The relative intensities of the signals would provide quantitation of the relative amounts of the protein in the different samples.

[0070] The technique is applicable to any sample containing an amine. For example; likely candidates include, but are not limited to cells, cellular extracts, sub-cellular extracts, cellular lysates, peptides, proteins, drugs, antibodies, pollutants and toxins.

[0071] The method can be used in a manner that does not affect post-translational modifications. The detection of post-translational modifications is important because numerous vital activities of proteins are regulated by post-translational modifications. For example, cell cycle progression, cellular differentiation, development, hormone response and protein kinase activation are all modulated by post-translational modifications.

[0072] The method can employ a wide range of separation techniques. For example, the method can employ 1-D gel electrophoresis, 2-D gel electrophoresis, zone electrophoresis, isotachopheresis, ion exchange chromatography, normal phase chromatography, reverse phase chromatography, hydrophobic interaction chromatography, size exclusion chromatography and combinations thereof.

Methods and materials

[0073] Any sample of amine-containing molecules can be quantified by the present invention. The most common samples envisioned are protein and peptide samples from cells, cellular extracts, sub-cellular extracts and cellular lysates. Samples may also include other peptides, proteins and drugs, antibodies, pollutants, and toxins.

[0074] The following methods will be described using protein samples as examples, but it is understood that any sample comprising amine-containing molecules may be used.

1) Labelling:

[0075] The method employs the reductive alkylation of amine groups to their alkylamine derivatives. Reductive alkylation is a convenient method for converting amino groups in proteins and peptides to their alkylamine derivatives through the reduction of the Schiff base that forms between the ϵ -amino group of lysine residues and an added aldehyde. In the case of formaldehyde (CH_2O) and sodium cyanoborohydride (NaCNBH_3) as a reducing agent for the Schiff base, free amino groups in proteins (mostly on sidechains of the amino acid lysine, but also the N-terminal amino group of the protein if this is not blocked) can be selectively and quantitatively converted to their dimethyl derivatives at slightly alkaline pH under very mild reaction conditions. The changes in the physical and chemical properties of the protein are minimal because of the small size of the introduced methyl groups (and

therefore minimal changes in molecular weight). The charge of the amino group is also retained with only a small alteration in the value of the isoelectric point, pI, so that the derivatized proteins will migrate in the two dimensions (molecular weight and pI) of 2-D gel electrophoresis almost exactly the same as the underivatized proteins.

[0076] In the case of a protein sample, the lysine residues can be derivatized with an aldehyde (for example formaldehyde, CH_2O) and a reducing agent (for example sodiumcyanoborohydride, NaCNBH_3), in the presence of an alkaline buffer ($\sim \text{pH } 8$). The lysine residues representing a second sample can be derivatized with an isotopically labelled aldehyde (for example CD_2O), and the same reducing agent. Lysine residues representing other samples are derivatized with other isotopically labelled aldehydes (or acetaldehydes) and a reducing agent (NaCNBH_3). The number of samples that can be labelled can be further increased by the use of isotopically labelled reducing agents (for example, NaCNBD_3) instead of NaCNBH_3 to introduce different degrees of deuterium labelling. The tags are as chromatographically similar as possible but with a large enough mass shift to be able to be easily distinguished by a mass spectrometer.

[0077] The specific, optimal pH conditions for reductive alkylation are determined by the properties of the analyte. For example, in the case of proteins, conditions must be chosen to minimise cross-linking reactions. Specifically, the reducing agent should be added first and at a $\text{pH} < 7$. This also minimises the formation of N-cyanomethyl adducts from impurities in the NaCNBH_3 . However, too low a pH reduces the reaction efficiency since the pK_a of ϵ -amino groups is ~ 11 . At $\text{pH } 6.5$, alkylation of the α -amino is favoured. Similarly, when NaCNBH_3 is used, reaction efficiency decreases above $\text{pH } 8$. Therefore, for complete methylation of proteins, a pH of 7-8 is recommended.

[0078] For other amino compounds, reductive alkylation is typically performed in organic solvents in the presence of 10-30% acetic acid. These amines are generally weaker bases than amines in proteins and, therefore, do not lose reactivity at low pH. In such cases, the acid is beneficial in that it accelerates the dehydration after reaction between the aldehyde and the amine.

[0079] Any aldehyde can be used. Typically, formaldehyde or acetaldehyde is used. Other aldehydes can be used, however, they do not react quantitatively and so are not ideal for protein analysis. Other aldehydes may be suitable for small molecules where more extreme reaction conditions can be used to promote a quantitative reaction. Although, larger aldehydes tend to be less reactive due to steric hindrance.

[0080] Schiff base reducing agents that can be used include: sodium cyanoborohydride and sodium borohydride. However, sodium borohydride is very reactive, and can further reduce the aldehyde to an alcohol. Other reducing agents can be used. For example, alkylborane or pyridine borane complexes can also be used for the reduction.

[0081] Optionally, a denaturing agent can be added to unfold the protein as is known to those skilled in the art in order to label buried lysine residues.

[0082] The reactants can be added in any order under almost any conditions. Preferably, however, the aldehyde is combined with the buffer and then mixed with the sample in the presence of the reducing agent. As is known to those skilled in the art, some care must be taken in using reaction conditions that prevent unwanted side-reactions such as crosslinking of the protein by the aldehyde.

[0083] As explained above, many different samples can be compared using different isotopically labelled reagents. Isotopic labelling techniques are known to those skilled in the art. For example, Table 1 lists differentially labelled formaldehyde and sodium cyanoborohydride reagents that can be used to differentiate samples.

[0084] Table 1 Differentially labelled formaldehyde and sodium cyanoborohydride.

Formaldehyde	Reducing Agent	Solvent	Added Mass per NH₂	Δ Mass per NH₂
CH ₂ O	NaCNBH ₃	H ₂ O	28.0316	0.0000
CH ₂ O	NaCNBD ₃	D ₂ O	30.0474	2.0158
¹³ CH ₂ O	NaCNBH ₃	H ₂ O	30.0316	2.0000
¹³ CH ₂ O	NaCNBD ₃	D ₂ O	32.0474	4.0158
CD ₂ O	NaCNBH ₃	H ₂ O	32.0632	4.0316
CD ₂ O	NaCNBD ₃	D ₂ O	34.0790	6.0474
¹³ CD ₂ O	NaCNBH ₃	H ₂ O	34.0632	6.0316
¹³ CD ₂ O	NaCNBD ₃	D ₂ O	36.0790	8.0474

[0085] Although the preferred method of labelling amine groups is described above, the invention encompasses the use of other classes of amine reactive reagents. For example:

a) Isocyanates: Isotopic reagents consisting of isocyanates are very reactive toward amino groups. They may not be as stable as the aldehyde and Schiff base reaction described above, and may decompose during storage.

b) Isothiocyanates: Isotopic reagents consisting of isothiocyanates can also be used. They are generally less reactive than aldehydes and may hydrolyse in aqueous solution.

c) Succinimidyl esters: Isotopic reagents consisting of succinimidyl esters may also be used, although they are less reactive than aldehydes and tend to hydrolyse in aqueous solutions, especially at alkaline pH (> pH 9). Further, reactivity can be low for reagents that are not highly soluble in aqueous solution.

d) Sulfonyl halides: Isotopic reagents consisting of sulfonyl halides can be used but are generally quite unstable in aqueous solution, especially at pH values necessary for reactivity of amino groups.

e) Other aldehydes and ketones: Isotopic reagents consisting of higher aldehydes and ketones are possible but they may generally provide lower reactivity due to poor solubility and/or steric hindrance at the reactive carbonyl group.

[0086] In addition, other groups (other than amines) can be targeted on proteins. For example:

a) Thiol-disulfide: In proteins, thiol groups are present in cysteine residues. Generally, thiols exist in the oxidized cysteine form and must be reduced to the free thiol for reactivity. The thiol-reactive reagents generally fall into one of the following classes, iodoacetamides, maleimides, benzylic or aliphatic halides. Iodoacetamides are generally quite reactive toward thiols and form stable products but the reagents may be unstable in light, especially in solution. Maleimides are generally selective reagents for thiol modification but they can suffer from poor solubility and significant hydrolysis to the corresponding maleimic acid, especially above pH 8.

b) Alcohols: In proteins, alcohol groups are present in the form of serine, threonine and tyrosine residues as well as carbohydrate residues of glycoproteins. However, few reagents are selective for the hydroxyl group in aqueous solution, especially in the presence of the more nucleophilic amino and thiol groups. Furthermore, any reagent that would be reactive toward hydroxyl groups may be readily hydrolysed in aqueous solution.

c) Carboxylic acids: In proteins, carboxylic acids are present in aspartic and glutamic acid residues. These residues can be coupled to hydrazines and amines in aqueous solution with the aid of water-soluble carbodiimides as activating agents. In order to minimize side reactions such as intra- and inter-protein coupling via lysine residues, these reactions must be conducted in concentrated protein solutions and at low pH, using a large excess of the reagent.

2) Combining samples and optional separation:

[0087] The different protein samples are combined. Optionally, for complex samples, the proteins are separated by methods known in the art, including 1D or 2D gel electrophoresis. Other methods for protein separation include: various modes of chromatography, including, ion-exchange, normal phase, reverse phase, hydrophobic interaction, size exclusion, various

modes of electrophoresis, including zone, gel and isotachopheresis as well as combinations of any of the above methods. After separation of the proteins, the gel is stained by one of several established methods known in the art, for example silver, Coomassie Blue or metal based reverse staining. Since the different protein samples have been labelled with the same reagent, the behaviour during electrophoresis of a particular protein should be identical regardless of the isotopic label employed. Upon staining of the gel with a reagent such as silver nitrate, each band/spot contains a particular protein from each of the samples. The stained protein bands/spots are then excised.

3) Enzymatic cleavage of the sample to yield fragments:

[0088] The samples are enzymatically cleaved to generate peptide fragments from the protein in the different samples. Although the conversion of the ϵ -amino group to a dimethylamino group renders the lysine residues unreactive to trypsin, the enzyme that is most commonly used to generate peptides for mass spectrometric analysis, trypsin will cleave a derivatized protein at the arginine residues (whose abundance in proteins is similar to that of lysine). Therefore, it is possible to perform tryptic digestion of labelled proteins and obtain accurate quantitative results using the disclosed method. Further, the proteins can also be cleaved by other enzymes such as chymotrypsin or by chemical means. However, the collision induced dissociation (CID) fragmentation of peptides generated by these methods is not as facile as for tryptic peptides. Computer databases exist for sequencing of proteins from the chymotryptic peptides.

4) Separation of fragments and introduction into mass spectrometer:

[0089] The peptide fragments are then introduced into the mass spectrometer, preferably using peptide separation methods known to those skilled in the art, including liquid chromatography or capillary electrophoresis. Preferably, the method is HPLC. The peptides can also be introduced without separation using electrospray ionization methods such as nanospray, pneumatically assisted electrospray, ionspray and turboionspray.

5) Examining the fragments by mass spectrometry:

[0090] The fragments are then examined by mass spectrometry. In this step both the quantity and sequence identity of the proteins from the gel spot can be determined. Peptides

from the different samples are quantified by measuring the relative signal intensities for the ions of identical sequence that are tagged with different isotopic forms of the reagents. For example, the peptides labelled with CD_2O and NaCNBH_3 prior to separation-digestion would have a molecular mass 4 Da higher than peptides from the same protein that were labelled with CH_2O and NaCNBH_3 . For some commonly used enzymes in the cleavage step, the peptides show up in the mass spectrum predominantly as doubly-charged ions so the mass to charge spacing is half of the molecular mass spacing. The appearance in the mass spectrum of a doublet separated by 4 Da (molecular mass) is indicative of the protein from the two different samples. If a third sample is labelled with $^{13}\text{CH}_2\text{O}$ and NaCNBH_3 and run on the same gel, a triplet in the mass spectrum with a separation of 2 Da occurs.

[0091] Many different mass spectrometers can be used in this invention. They include:

- (i) Fourier transform – Ion cyclotron resonance mass spectrometers (FT-ICR-MS)
- (ii) Time of Flight mass spectrometers (TOF-MS, TOF-TOF-MS)
- (iii) Ion trap mass spectrometers (IT)
- (iv) Quadrupole mass spectrometers (Q-MS and QqQ-MS)
- (v) Ion mobility mass spectrometers (IM-MS)

Hybrid-geometry mass spectrometers can also be used for the invention, examples include:

- (vi) Quadrupole (or hexapole, octapole)-Time of Flight mass spectrometers (Q-TOF, and Qq-TOF)
- (vii) Ion trap – Time of flight mass spectrometers (IT-TOF)

[0092] The above mass spectrometers can be combined with ionization sources such as electrospray ionization at any flow rate, matrix-assisted laser desorption and ionization (MALDI), field desorption, thermal desorption and laser desorption from suitable substrates (i.e. titanium dioxide and silicon dioxide).

6) Analyzing the fragments and Sequencing the fragments:

[0093] Sequencing is an optional step. Sequencing information is generated by selecting peptide ions of a particular mass-to-charge ratio for collision-induced dissociation (CID) in the mass spectrometer operating in the MS/MS mode. Using sophisticated computer-searching algorithms, the resulting CID spectra is automatically correlated with sequence databases to identify the protein from which the sequenced peptide originated. Other modes of analysis include peptide mass fingerprinting, peptide mapping, Edman sequencing and sequencing by sequential amino acid cleavage.

7) Kits:

[0094] The present invention encompasses kits comprising isotopically labelled reagents, protocols and software for the method of quantitatively analyzing a sample. The reagents include multiple sets of isotopic reagents for labelling multiple samples, buffers and isotopic solvents for conducting labelling reactions. The software contains algorithms for detecting multiplets of peaks corresponding to groups of sample peaks from a plurality of samples. Algorithms are also included for converting the intensity of the spectra to relative intensity of sample components. Protocols are included for extraction of proteins from cells, reaction of proteins and methods for sample clean-up.

Examples

[0095] The invention is further described by the following examples.

Example 1: Quantitative analysis of protein samples differentially labelled by isotopic chemical derivatization.

Materials:

[0096] Formaldehydes, protein standards and other reagents except where noted were obtained from Sigma-Aldrich Chemical Co. (Mississauga, ON). Deuterium oxide (99.9%) was obtained from Cambridge Isotope Laboratories (Woburn, MA). All solutions were

prepared in deionized double distilled water. Bulk reversed phase column packing material (Poros 50 R1) was obtained from PerSeptive Biosystems.

Methods:

[0097] i) Protein labelling: Protein standards were dissolved in water or 0.1% acetic acid (when necessary) to an initial concentration of 100 pmol/ μ L. Appropriate aliquots of each solution were combined and the sample was lyophilized. The samples were then treated with equal volumes of freshly prepared 1M formaldehyde and 1M sodium cyanoborohydride in 50 mM NaH_2PO_4 (pH 8.5), in either H_2O or D_2O , to provide at least 100 fold excess of each reagent. Samples were permitted to react for 18 hours at room temperature with periodic mixing. Labelled protein samples were then stored at 4°C until cleanup.

[0098] The reason that deuterated water was used is that the reducing agent, NaCNBD_3 , can under certain conditions, react with water to form NaCNBH_3 . This reducing reagent would not produce the correct mass tag. Therefore, the use of deuterated water (or another deuterated solvent) is an important reaction condition when using deuterated reducing agents.

[0099] ii) Sample Mixing: Prior to mixing of the isotopically labelled samples, sufficient 2M ammonium bicarbonate was added to each sample to react with any excess formaldehyde. This step is not necessary but it is recommended since excess reagent can react with amine containing buffers, such as TRIS, used in subsequent sample processing steps. After 30 minutes, the appropriate volume of each sample was combined.

[00100] iii) Gel Electrophoresis: Optionally, combined samples were subjected to SDS-PAGE using standard conditions which included a 4% stacking gel and a 12% resolving gel in Tris-Glycine buffer systems. After electrophoresis, proteins were visualized by staining methods using either silver nitrate or Coomassie Blue. Protein bands were excised and subjected to in-gel cysteine carbamidomethylation with DTT/iodoacetamide using published procedures.

[00101] iv) Sample Cleanup: Optionally, when samples were not subjected to SDS-PAGE, the buffer was exchanged before enzymatic digestion of the proteins. This was

accomplished using reverse phase (C₄) columns that were laboratory-prepared in gel-loading pipet tips according to published procedures. Samples were passed three times through columns that had been previously conditioned with methanol followed by 0.1% trifluoroacetic acid (TFA) in water. The column was then washed with 50 μ L of aqueous 0.1% TFA and the proteins were eluted with 85% acetonitrile, 0.5% formic acid (2 x 50 μ L) and lyophilized.

[00102] v) Enzymatic Digestion: Proteins larger than approximately 5 kDa could be hydrolyzed before being examined by MS. This is usually done using an enzyme, however, one could also use chemical methods for digestion using, e.g. CNBr or acid (HCl, formic acid). In this example, sequencing-grade chymotrypsin (TLCK-treated) was dissolved in 1mM HCl at 5 ng/ μ L and diluted 400 fold in 0.1M ammonium bicarbonate. Lyophilized gel bands were covered with the chymotrypsin solution, typically 25-40 μ L. Reverse phase cleanup samples were dissolved in 25 μ L of the enzyme solution. Digestions were conducted for 24 hours at 37°C. After digestion, gel bands were extracted with 50% acetonitrile containing 0.5% formic acid, according to established procedures. Extracts were lyophilized and stored at 4°C prior to analysis by MS.

[00103] The results of the protein analysis can be found in Figures 1 and 2.

[00104] Figure 1 is a MS spectrum of the same protein from two samples, where each sample was labelled with a unique tag, and digested with chymotrypsin and analysed using ESI-MS. There are two doubly-charged peptides. In this example, the monoisotopic masses of the $[M+H]^+$ of the derivatized peptides are 1255.794 and 1259.780.

[00105] Figure 2 is a MS spectrum as in Figure 1, except that three samples were labelled. In addition the peptides were triply charged. The spacing of the tags is small enough to minimize the difference in chromatographic properties, but large enough to allow accurate quantitation of even small changes in protein expression. It would not be possible to quantify such small differences in protein levels using conventional staining techniques, such as silver staining.

Example 2: Analysis of doubly labelled in-gel digest of BSA.

[00106] Following the method of Example 1, BSA was labelled and analysed by LC-MS. Figure 3 is a LC- MS spectrum demonstrating that the tags were compatible with gel electrophoresis and also demonstrating the added specificity obtained through labelling. Any peak that appeared as a doublet was from the sample and was analysed. The singlet peaks were due to trypsin autolysis products, keratin contamination or sample peptides lacking lysine.

Example 3: Analysis of isotopically labelled peptides by Tandem MS.

[00107] Following the method of Example 1, two peptides were isotopically labelled and analysed by tandem MS spectrum. Figure 4 shows the tandem MS spectrum. Both labelled peptides were fragmented at the same time. This resulted in the appearance of doublet peaks for fragments containing the label (fragments b_6 — b_{11} and y_1 — y_7). The tag did not interfere with the ability to perform sequencing experiments through collision-induced fragmentation (CID). The CID process followed a similar pattern to that observed with non-labelled peptides.

Example 4: Analysis of isotopically labelled peptides by HPLC-MS.

[00108] Following the method of Example 1, peptides were isotopically labelled and analysed by HPLC-MS. Figure 5 is a composite of 4 spectrums that show the added complexity of resolution of peaks by HPLC-MS. Top left, nanoelectrospray spectrum; bottom left, total-ion chromatogram for the m/z range of 475-485; top right, MS spectrum for the first peak consisting of a triplet of doubly charged peptides; bottom right, MS spectrum for a second peak consisting of a triplet to triply charged peptides.

Example 5: Analysis of two samples of differentially labelled BSA.

[00109] Two samples of BSA were differentially labelled, combined, digested using trypsin and analysed by LC-MS. Figure 6 shows the resulting MS spectrum and ion chromatogram. Trace A shows the MS spectrum of a pair of labelled tryptic peptides and Trace B shows the extracted ion chromatograms for the same two peptides. The data demonstrates that the labelling did not affect the ability to use trypsin for digestion of the labelled proteins and, furthermore, that the digestion produced doubly charged peptides in

the mass range that is suitable for low-energy CID (1000-3000 Da) and subsequent database searching. The data also demonstrates that the labelled peptides eluted at the same time and were chromatographically indistinguishable using standard HPLC conditions. The fact that the labelled peptides co-eluted is ideal for accurate quantitation.

Example 6: Analysis of membrane proteins from two samples of *Aeromonas salmonicida* grown under different conditions.

[00110] Two samples of *Aeromonas Salmonicida* were grown under different conditions. The first sample was grown using standard cell-culture conditions and the second sample was grown as a biofilm in minimal media. The two samples were differentially labelled and then separated by 2D Gel electrophoresis as described in Example 1 and shown in Figure 7. Panel A is a separation of membrane proteins from one sample of the bacteria *Aeromonas Salmonicida*. Panel B is a separation of the two samples of *Aeromonas Salmonicida* grown under different conditions and differentially labelled. The proteins were extracted from cultured bacteria and the separation was performed using isoelectric focusing (pH 3-11) for approximately 12 hours followed by SDS-PAGE on a 12% acrylamide gel. The gels were stained with silver. This example demonstrates the compatibility of the differential labelling technology for processing multiple samples in the same gel.

Example 7: Analysis of various differentially labelled amines.

[00111] Various amines were labelled with either CH_2O or CD_2O and reduced with sodium cyanoborohydride or sodium cyanoborodeuteride in acetonitrile which contained 10% (v/v) acetic acid or acetic acid d_4 . Various amounts of each sample were mixed and analysed by LC-MS. No digestion was required, as the molecules were small. The MS used was a triple-quadrupole instrument (API III+) with an IonSpray source operated in the positive-ion mode. Table 2 provides the details of the analysis. Column 1 lists the names of the amines. Column 2 lists the mass of the protonated pseudo-molecular ion. Columns 3 and 4 list the amount of differentially labelled amine combined for the analysis. Columns 5 and 6 list the expected and experimentally determined ratios, respectively. Finally, column 7 lists the calculated percent error. Figure 8 shows the molecular structure of the amines.

The lower panel shows the mass spectrum of 3-aminothiophenol labelled with CH_2O ($m/z = 123.0$) and CD_2O ($m/z = 127.0$) and NaCNBH_3 . The expected ratio of intensities was 1.07 and the observed ratio was 1.10, corresponding to an error of 2.7%.

Table 2 Analysis of various amines

Compound	[M+H] ⁺ (Da)	mass of CH_2O labelled compd (mg)	mass of CD_2O labelled compd (mg)	Expected Ratio	Observed Ratio	% error
3-Aminopyridine	95.1	52.5	56.1	0.94	0.88	-5.8
Aminoacetaldehyde diethylacetal	134.2	59.3	54.9	1.08	0.98	-9.3
3-Aminophenol	110.1	52.1	48.0	1.09	1.01	-6.8
Anthranilic acid	138.2	49.4	58.9	0.84	0.77	-8.7
3-Aminothiophenol	126.2	51.3	48.0	1.07	1.10	2.7
Benzylamine	108.2	42.9	55.3	0.78	0.66	-14.8
Morpholine	88.1	57.0	57.2	1.00	0.98	-1.9
Tryptophan	205.2	42.8	50.9	0.84	1.00	18.7
Kainic acid	214.2	39.6	44.4	0.89	0.95	6.9
		40.3	13.2	3.05	2.95	-3.2
		11.5	56.1	0.20	0.24	16.6
Domoic acid	312.2	39.6	44.4	0.89	1.05	17.6
		40.3	13.2	3.05	3.34	9.5
		32.3	68.6	0.47	0.51	7.4
3-Amino-5-phenyl- Pyrazole (APPY)	160.2	32.3	68.6	0.47	0.51	7.4
2-(4-Aminophenyl)- 6-methylbenzothiazole (AMBT)	241.2	28.6	60.0	0.48	0.42	-12.9

[00112] The data demonstrates that the labelling is compatible with primary amines, including aliphatic compounds (1), aromatic compounds (2)-(5), (10)-(12), and secondary amines (7)-(9). Further, the aromatic heterocyclic nitrogen atoms are not methylated whereas the aliphatic nitrogens are labelled. The compounds in (8) and (9) are representative of food toxins, and the compounds of (10)-(12) are representative of drugs.

Example 8: Analysis of a plurality of differentially labelled amine containing molecules.

[00113] In this example, four samples of 3-aminopyridine were labelled by the method described in Example 7. The samples were analyzed by MALDI-MS. The data are displayed in Figure 9. The peaks at m/z 122.98, 124.99, 127.01 and 129.02 correspond to

the labelled amines from the various samples. The expected ratio was 1:1.06:0.43:0.2 and the ratio obtained was 1:1.03:0.62:0.25.

[00114] Although the invention has been described with examples, it is to be understood that modifications may be resorted to as will be apparent to those skilled in the art. Such modifications and variations are to be considered within the purview and scope of the present invention.

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Claims

What is claimed is:

1. A method for the simultaneous quantitative analysis of a plurality of samples of amine-containing molecules, comprising:
 - (i) reacting the samples with isotopically labelled reagents wherein the reagents react with the amines, resulting in isotopically labelled derivatives;
 - (ii) combining the samples; and
 - (iii) examining the samples by mass spectrometry.
2. A method for the quantitative analysis of a sample of amine-containing molecules, comprising:
 - (i) reacting the sample with isotopically labelled reagents resulting in the reductive alkylation of the amines to their alkylamine derivatives, such that the alkylamine derivatives are isotopically labelled; and
 - (ii) examining the sample by mass spectrometry.
3. A method for the quantitative analysis of two or more samples of amine-containing molecules, comprising:
 - (i) reacting the samples with isotopically labelled reagents resulting in the reductive alkylation of the amines to their alkylamine derivatives, such that the alkylamine derivatives are isotopically labelled;
 - (ii) combining the samples; and
 - (iii) examining the samples by mass spectrometry.
4. The method of claim 3 further comprising a step of enzymatically cleaving the samples into fragments, prior to examining the samples by mass spectrometry.
5. The method of claim 1, 2 or 3 comprising an additional step of denaturing the samples prior to reacting the samples with isotopically labelled reagents.

6. The method of claim 1, 2, or 3 wherein the step of analysing the fragments by mass spectrometry comprises introducing the fragments to a mass spectrometer using electrospray ionisation.
7. The method of claim 6 wherein the electrospray ionisation method is selected from a group consisting of nanospray, pneumatically assisted electrospray, ionspray and turboionspray.
8. The method of claim 1, 2 or 3 comprising an additional step of separating the amine-containing molecules in the samples before analyzing the samples.
9. The method of claim 8 wherein the step of separating the amine-containing molecules uses a separator selected from a group consisting of 1-D gel electrophoresis, SDS-PAGE, isoelectric focusing, 2-D gel electrophoresis, zone electrophoresis, isotachopheresis, ion exchange chromatography, normal phase chromatography, reverse phase chromatography, hydrophobic interaction chromatography, size exclusion chromatography and any combination of these separators.
10. The method of claim 3 comprising an additional step of separating the fragments after enzymatically cleaving the fragments.
11. The method of claim 10 wherein the step of separating the fragments uses a separator selected from a group consisting of liquid chromatography, high performance liquid chromatography and capillary electrophoresis.
12. The method of claims 1, 2 or 3 comprising an additional step of analyzing the fragments after examining the fragments by mass spectrometry.
13. The method of claim 12 wherein the step of analyzing the fragments is selected from a group consisting of collision-induced dissociation in a mass spectrometer operating in MS/MS mode, peptide mass fingerprinting, peptide mapping, Edman sequencing and sequencing by sequential amino acid cleavage.
14. The method of claim 13, comprising an additional step, after the step of analyzing the fragments, of using computer-searching algorithms to correlate the data with

sequences listed in sequence databases to identify the molecule from which the fragment originated.

15. The method of claim 1, 2 or 3 wherein the isotopically labelled reagents are an aldehyde and a reducing agent.
16. The method of claim 15 wherein the aldehyde is selected from a group consisting of formaldehyde and acetaldehyde.
17. The method of claim 16 wherein the reducing agent is selected from a group consisting of a sodium cyanoborohydride, sodium borohydride, dialkyl borane complexes and pyridine borane complexes.
18. The method of claim 1 wherein the sample is selected from a group consisting of cells, cellular extracts, sub-cellular extracts, cellular lysates, peptides, proteins, drugs, toxins, antibodies and pollutants.
19. The method of claim 18 wherein the proteins are extracted from cells.
20. The method of claim 19 wherein the amines of the proteins are selected from a group consisting of lysine residues, ornithine residues and residues at the N-terminal amino group of the proteins.
21. The method of claim 1, 2 or 3 wherein the method of analyzing the fragments by mass spectrometry utilizes a mass spectrometer selected from a group consisting of:
 - (i) Fourier transform – Ion cyclotron resonance mass spectrometers (FT-ICR-MS);
 - (ii) Time of Flight mass spectrometers (TOF-MS, TOF-TOF-MS);
 - (iii) Ion trap mass spectrometers (IT);
 - (iv) Quadrupole mass spectrometers (Q-MS and QqQ-MS);
 - (v) Ion mobility mass spectrometers (IM-MS);
 - (vi) Quadrupole (or hexapole, octapole)-Time of Flight mass spectrometers (Q-TOF, and Qq-TOF); and
 - (vii) Ion trap – Time of flight mass spectrometers (IT-TOF).

22. The method of claim 21 comprising an additional step of combining the mass spectrometer with an ionisation source.
23. The method of claim 22 wherein the ionisation source is selected from a group consisting of electrospray ionisation, matrix-assisted laser desorption and ionisation (MALDI), field desorption, thermal desorption and laser desorption.
24. A plurality of samples of amine-containing molecules for simultaneous quantitative analysis by mass spectrometry, each sample comprising isotopically labelled derivatives resulting from isotopically labelled reagents having reacted with the amine-containing molecules.
25. A sample of amine-containing molecules comprising isotopically labelled derivatives resulting from the reductive alkylation of the amines to alkylamine derivatives by isotopically labelled reagents.
26. Two or more samples of amine-containing molecules comprising isotopically labelled derivatives resulting from the reductive alkylation of the amines to alkylamine derivatives by isotopically labelled reagents.
27. Use of a mass spectrometer for the analysis of a sample according to any of claims 1, 2 or 3.
28. A kit comprising isotopically labelled reagents, protocols and software for the method of quantitatively analyzing a sample of claims 1, 2 or 3.
29. A method for the quantitative analysis of two or more cellular extracts comprising amine-containing molecules, comprising:
 - (i) reacting the extracts with different isotopically labelled reagents resulting in the reductive alkylation of the amines to their alkylamine derivatives, such that the alkylamine derivatives are isotopically labelled;
 - (ii) combining the extracts;
 - (iii) separating the amine-containing molecules;
 - (iv) enzymatically cleaving the amine-containing molecules into fragments;
 - (v) separating the fragments;

- (vi) analysing the fragments by mass spectrometry; and
- (vii) sequencing the fragments.

Application number / numéro de demande: #2393726

Figures: 7

Pages: P.9

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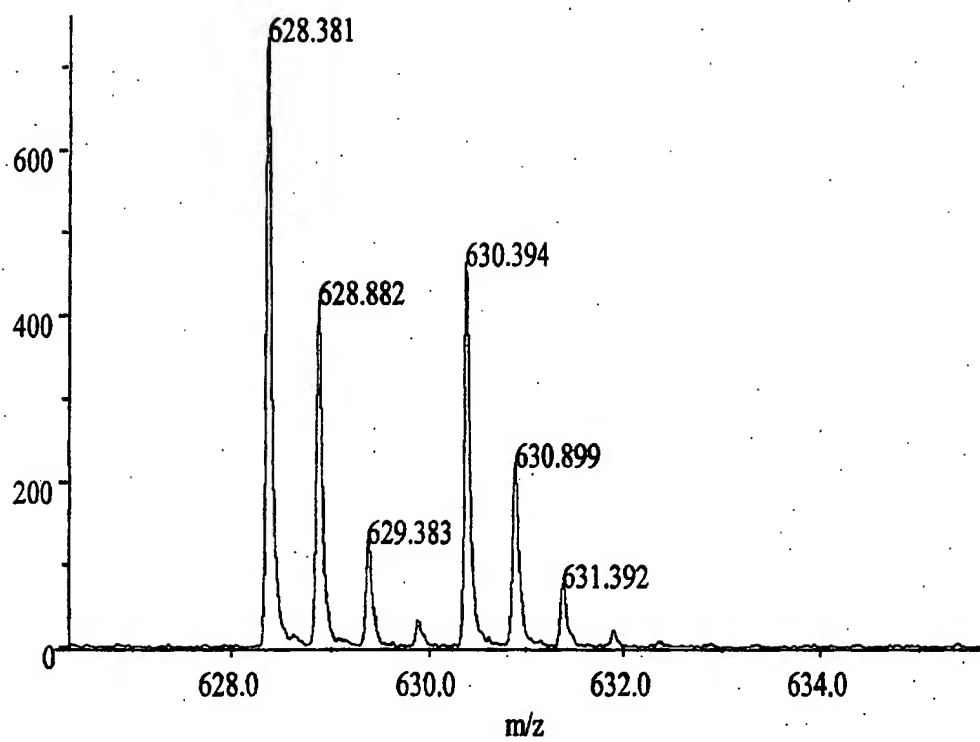


Figure 1

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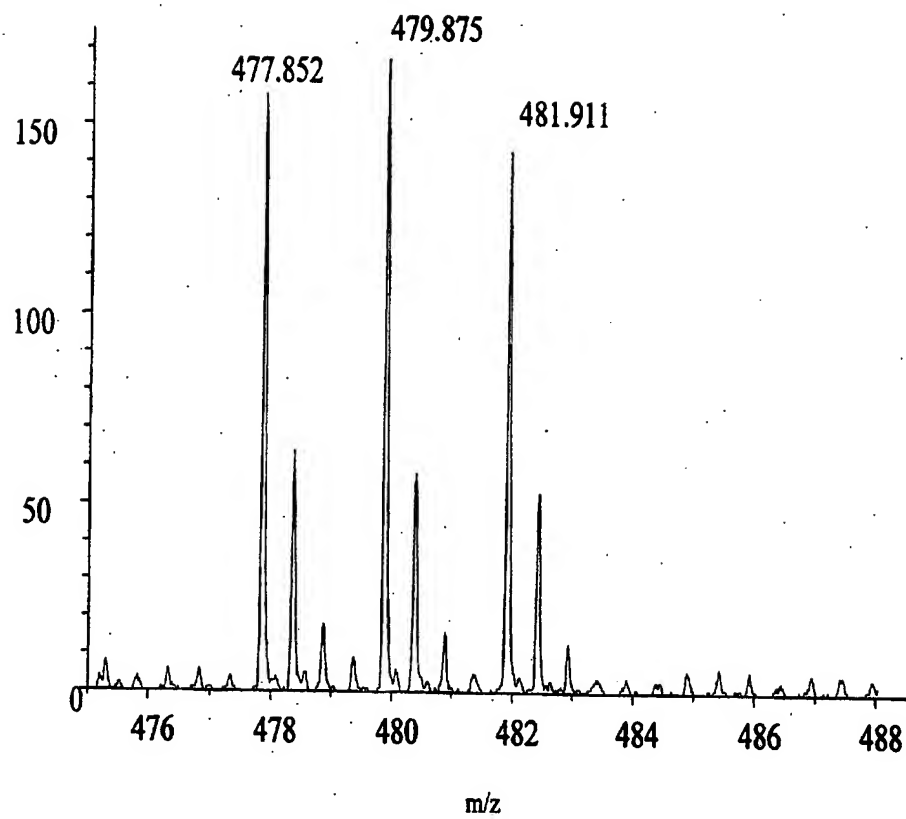


Figure 2

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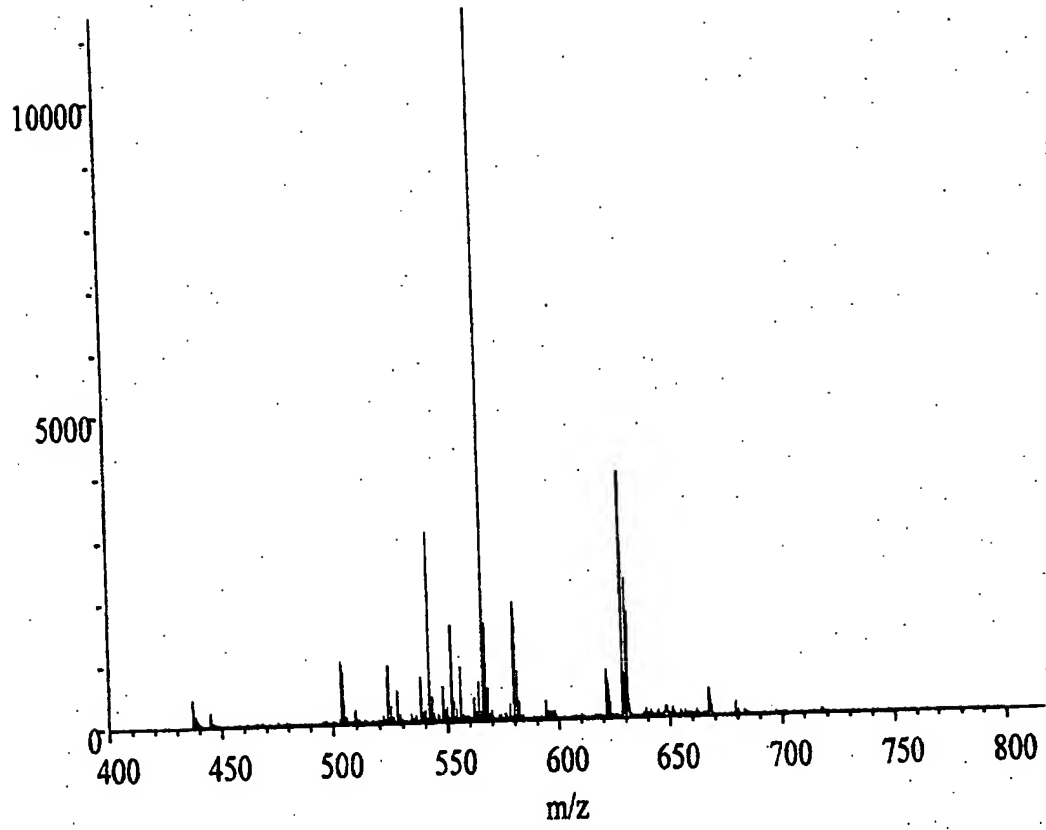
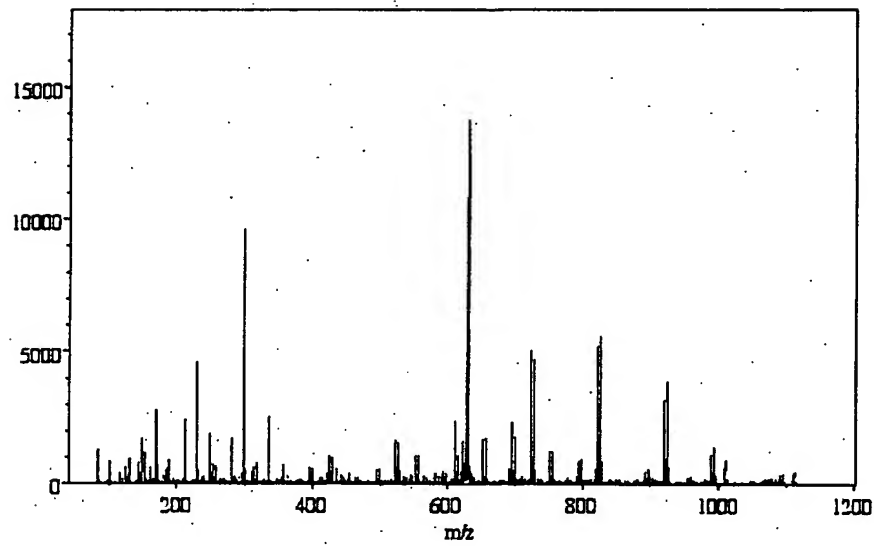


Figure 3

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A/V/E/G/P/J*/L/V/V/S/T/Q

Figure 4

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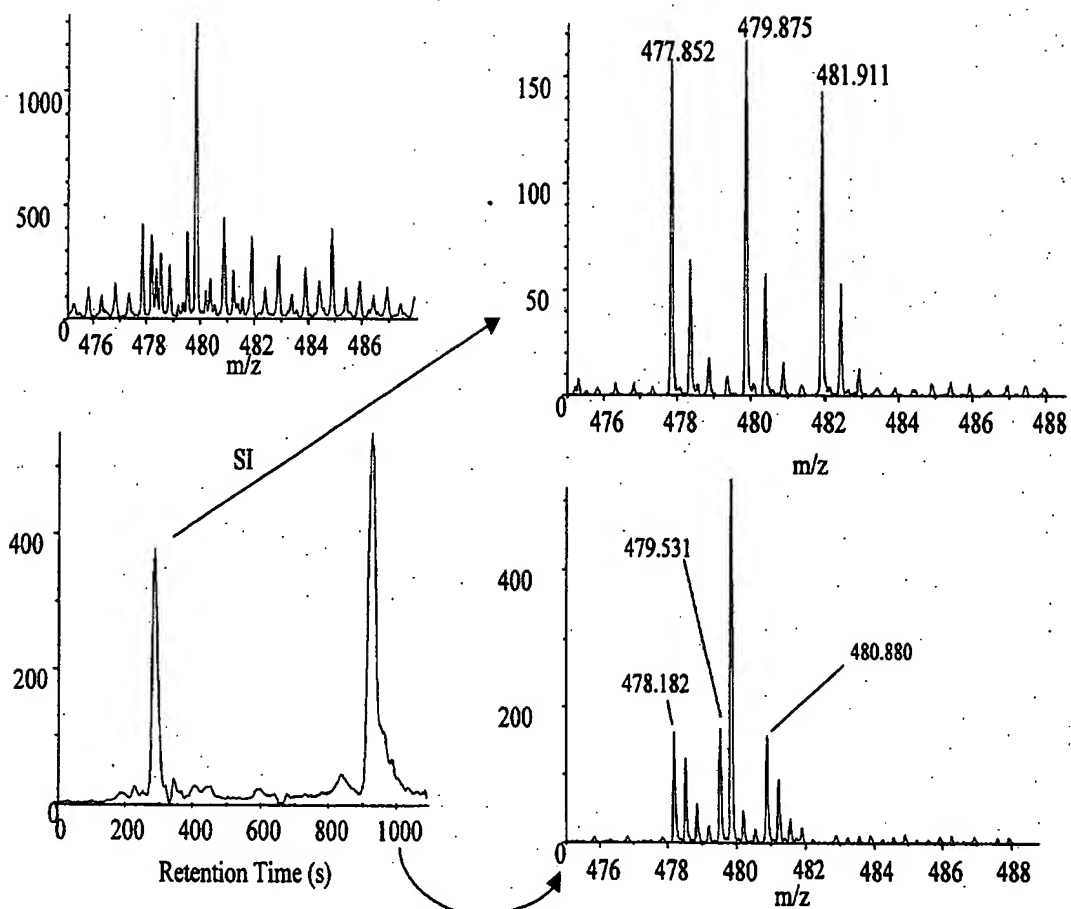


Figure 5

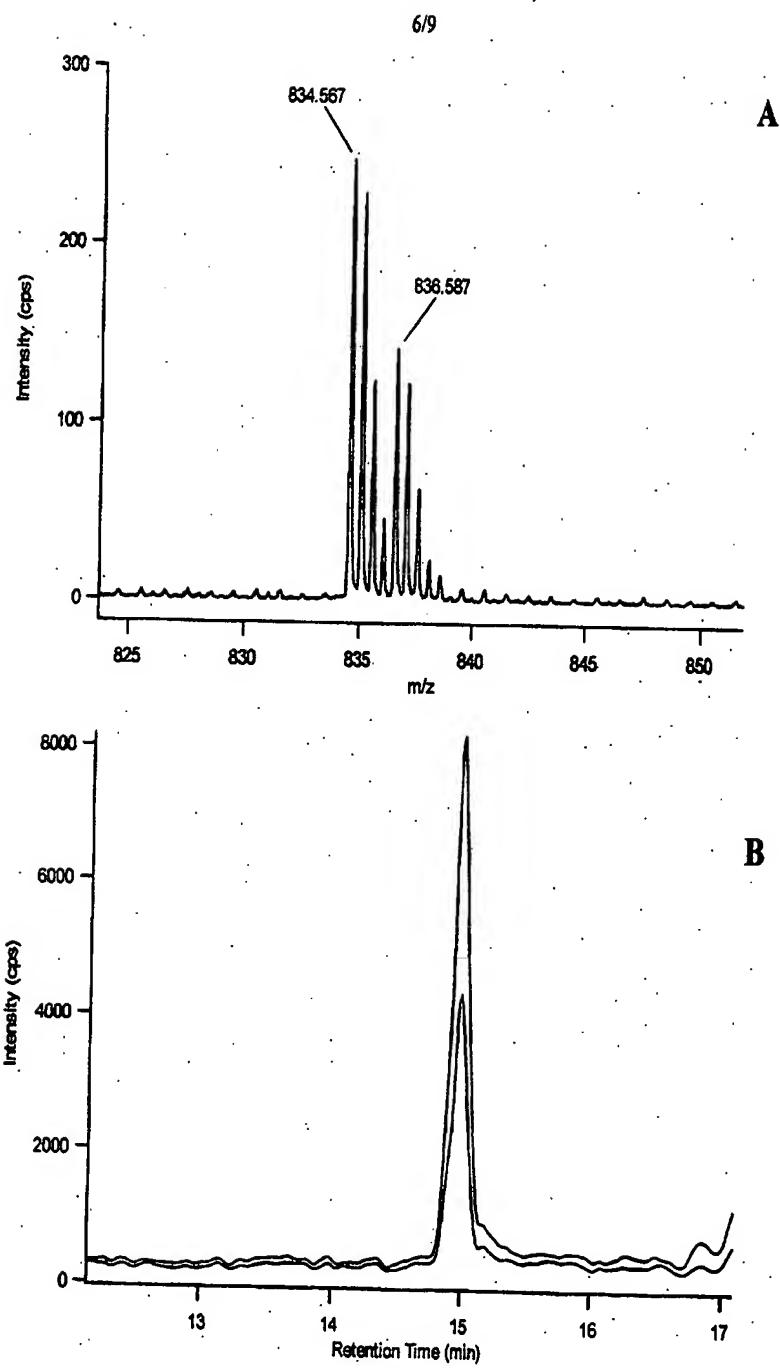


Figure 6

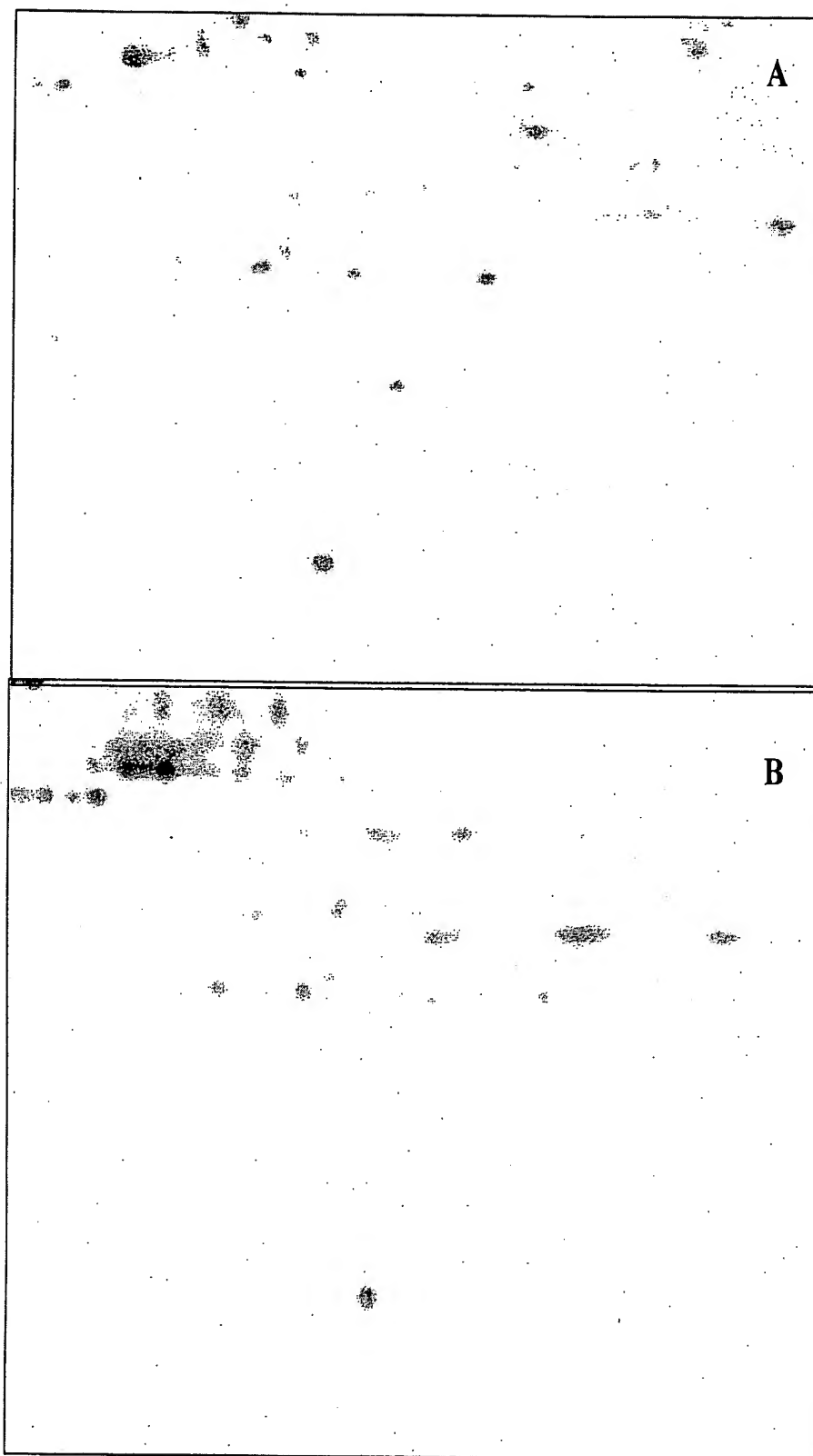
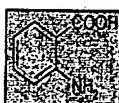


Figure 7

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(1) 3-Aminopyridine



(2) Anthranilic acid



(3) 3-Aminophenol



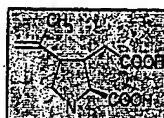
(4) Benzylamine



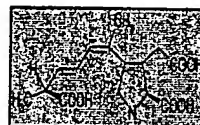
(5) 3-Aminothiophenol

(6) Aminoacetaldehyde
diethylacetal

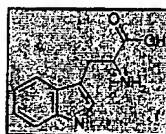
(7) Morpholine



(8) Kainic acid



(9) Domoic acid



(10) Tryptophan

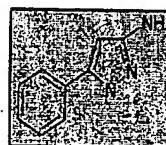
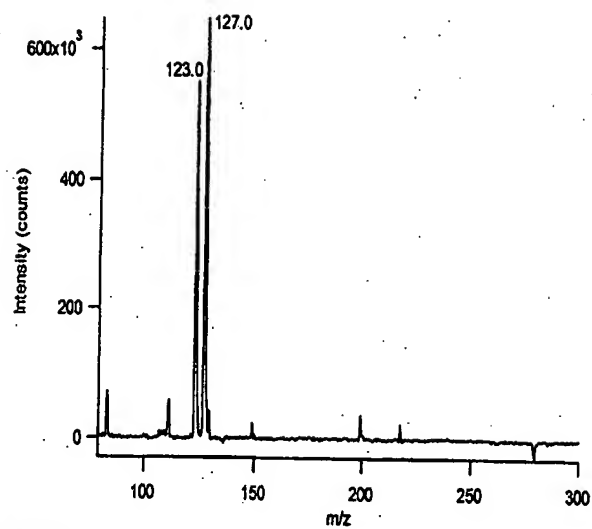
(11) 2-(4-Aminophenyl)-
6-methylbenzothiazole
(AMBT)(12) 3-Amino-5-phenyl-
pyrazole (APPY)

Figure 8

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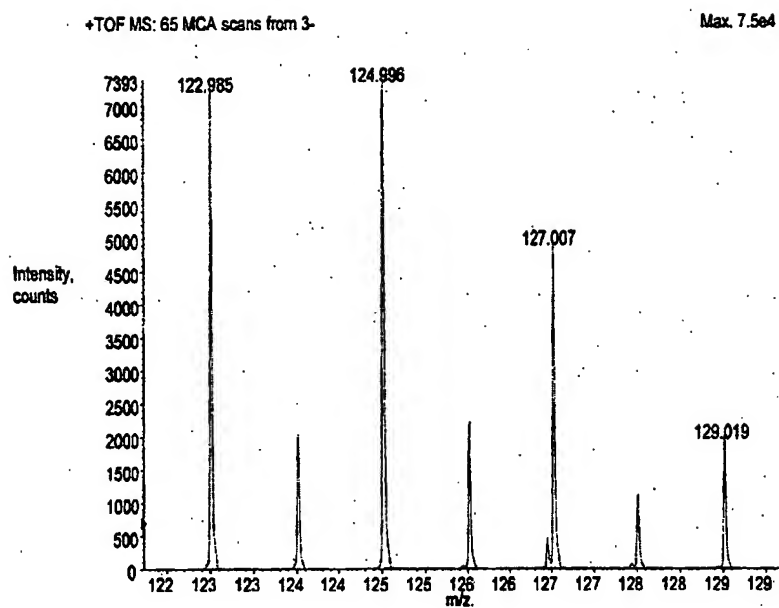


Figure 9